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Original Article **Open Access**

Discovery of potential antiviral compounds against Dengue virus using virtual screening and physicochemical profiling from zinc database

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Abstract:

Background: Dengue, a mosquito-borne virus infection, reportedly affected over 7.6 million people in early 2024, subsequently leading to approximately 3000 deaths. Dengue is primarily spread by *Aedes* (*A. aegypti and A. albopictus*) mosquitoes. There is currently no clinically approved preventive vaccine against Dengue virus (DENV), and according to the Center for Disease Control (CDC), the only acceptable vaccine for DENV is Dengvaxia, which has efficacy and safety issues. This makes it unsuitable for use against DENV. Drug repurpose is an alternative approach for the discovery of novel therapeutics.
Methodology: In this study, we screened over one million compounds in the zinc database against the DENV NS2B/NS3 protease (PDB ID: 2FOM). Screening yielded about 122 compounds with potential activity against DENV. Among the 122 compounds, the top four were selected based on their docking scores and commercial availability. The cytotoxicity study showed that a concentration of up to 100 µM was non-toxic. Based on the cytotoxicity results, we selected non-toxic concentrations (500nM, 1µM, 10µM, and 20µM) to test against DENV2 infection. The results were analyzed using RT-qPCR and plaque assays.
Results: Compound treatment before DENV2 infection leads to a significant reduction in viral replication, which confirms the screening result. This gives a promising platform against DENV2 therapy.
Conclusion: This shows a significant step towards the discovery of an antiviral drug against the dengue virus.

Key words: Dengue virus; Antiviral compound; Repurposing; Molecular docking; Therapeutics

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Découverte de composés antiviraux potentiels contre le virus de la dengue par criblage virtuel et profilage physico-chimique à partir d'une base de données sur le zinc

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Résumé:

Contexte: La dengue, une infection virale transmise par les moustiques, aurait touché plus de 7,6 millions de personnes début 2024, entraînant environ 3000 décès. La dengue est principalement transmise par les moustiques *Aedes* (*Aedes aegypti* et *Aedes albopictus*). Il n'existe actuellement aucun vaccin préventif contre le virus de la dengue (DENV) approuvé cliniquement. Selon les Centres pour le contrôle et la prévention des maladies (CDC), le seul vaccin acceptable contre le DENV est le Dengvaxia, qui présente toutefois des problèmes d'efficacité et d'innocuité. De ce fait, il est inadapté à la lutte contre le DENV. Le repositionnement de médicaments constitue une approche alternative pour la découverte de nouvelles thérapies.
Méthodologie: Dans cette étude, nous avons criblé plus d'un million de composés de la base de données sur le

zinc contre la protéase NS2B/NS3 du DENV (identifiant PDB: 2FOM). Ce criblage a permis d'identifier environ 122 composés présentant une activité potentielle contre le DENV. Parmi ces 122 composés, les quatre meilleurs ont été sélectionnés en fonction de leurs scores d'amarrage moléculaire et de leur disponibilité commerciale. L'étude de cytotoxicité a montré qu'une concentration allant jusqu'à 100 µM était non toxique. D'après les résultats de cytotoxicité, nous avons sélectionné des concentrations non toxiques (500nM, 1µM, 10µM et 20µM) pour tester leur efficacité contre l'infection par le DENV2. Les résultats ont été analysés par RT-qPCR et par test de plages de lyse.

Résultats: Le traitement par le composé avant l'infection par le DENV2 induit une réduction significative de la réplication virale, confirmant ainsi les résultats du criblage. Ceci constitue une plateforme prometteuse pour le traitement du DENV2.

Conclusion: Ces résultats représentent une avancée significative vers la découverte d'un médicament antiviral contre le virus de la dengue.

Mots-clés: Virus de la dengue; Composé antiviral; Repositionnement; Modélisation moléculaire; Thérapeutique

Introduction:

Dengue virus (DENV) infection is regarded as one of the most common arthropod-borne diseases in the tropical and subtropical regions. Cases of DENV infection reported by the World Health Organization (WHO) are about 5.2 million in 2019 (1,2). The WHO has reported dengue to be one of the most rapidly spreading mosquito-borne viral infections worldwide. It is estimated that approximately 60% of the world's population is at risk of infection by the year 2080 (2). Dengue viruses spread to people through the bite of infected *Aedes* mosquitoes (*Aedes aegypti* or *Aedes albopictus*) (3).

There are four DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), with DENV 2 and 3 being the most virulent forms (4). All four serotypes can cause hemorrhage (5). DENV can cause illnesses ranging from asymptomatic to dengue hemorrhagic fever (DHF), dengue fever (DF), and dengue shock syndrome (DSS). Clinical manifestations of DENV infection include fever, rash, hemorrhagic symptoms, nausea, vomiting, and ocular pain (6).

Dengue virus is a member of the genus Flavivirus of the family Flaviviridae (7). DENV has a small icosahedral envelope (8, 9) with an 11 kb positive-sense single-stranded RNA (10). This encloses the three structural proteins, including the capsid (C), envelope (E), and membrane (M), as well as seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (11,12). The mature DENV particles had a diameter of approximately 50 nm (7). The surface is composed of a lipid bilayer that incorporates two transmembrane viral proteins to form a glycoprotein shell (13). The core contains a nucleocapsid formed by the viral RNA genome complex with the capsid protein. The glycoprotein shell contains 180 copies of the envelope (E) and membrane proteins (M or prM). DENV infection depends largely on the conformational changes in the M and E proteins brought about by the pH of the environment (14).

The crystal structure of the dengue virus NS2B-NS3 protease revealed a pocket that can potentially be occupied by various compounds. This protease is one of the major virulent factors of the virus. It is important in viral integration with the host genome. This opens a novel way to identify antiviral agents that are active against the early stages of dengue virus infection (15). Currently, there are no FDA-approved therapeutic drugs against dengue virus infection, and a potent vaccine platform is still in its infancy. Significant work on vaccine development has yielded a single licensed vaccine with strict restrictions on use (11).

The major aim of this pilot study was to verify the safety profile of selected compounds using a cytotoxicity assay and test their antiviral activity against DENV. For this experiment, compound 1 was selected for antiviral assay against DENV2 based on availability and highest docking score compared to other compounds.

Materials and method:

In-silico analysis:

The crystal structure of the DENV protein (PDB ID: 2FOM) was retrieved from the Protein Data Bank. The Protein Preparation Wizard in Maestro (Schrödinger) was used to clean and optimize protein structure. The following steps were carried out; (i) removal of water molecules and other heteroatoms irrelevant to binding, (ii) assignment of bond orders, and (iii) addition of missing hydrogens. Optimization of protonation states for amino acids, especially those involved in catalysis or those close to the ligand-binding site, was performed using PROPKA at pH 7.0. The protein structure was refined by energy minimization using the OPLS4 force field. Additionally, a grid box of 20 Å was generated around the active site to define the docking space and ensure accurate positioning of the ligands during docking simulations.

High-Throughput Virtual Screening (HTVS):

The prepared zinc compounds underwent an initial docking step using the High-Throughput Virtual Screening (HTVS) mode within Schrödinger's Glide module. This screening step allows rapid evaluation of large libra-

ries by focusing on the best-fitting compounds for the protein-binding site. Over 100,000 compounds with the best docking scores were selected for further analysis. This stage prioritizes speed and efficiency and provides rough estimates of the binding affinity.

Standard Precision (SP) Docking:

The compounds selected from the HTVS step were re-docked using the Standard Precision (SP) docking mode in Glide. SP docking involves more thorough exploration of the binding site and ligand conformations, yielding more accurate docking scores. This step reduced the number of compounds from 100,000 to 250 based on the binding affinity, protein-ligand interactions, and key docking parameters. Additionally, a visual inspection of the binding modes was performed to ensure that only the most promising ligands progressed to the next stage.

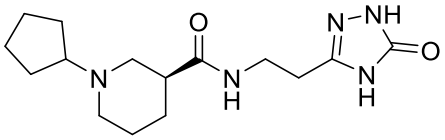
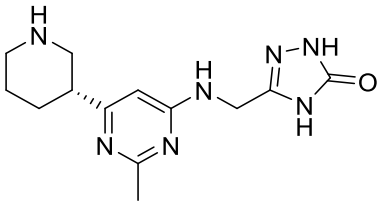
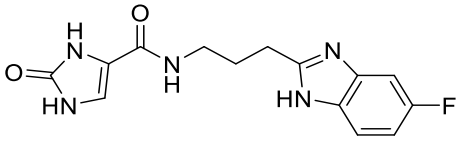
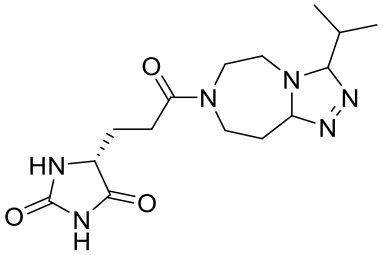
Extra Precision Docking (XP):

The 250 compounds from the SP step were subjected to Extra Precision (XP) docking. XP docking employs a more refined scoring function, penalizing unfavorable interactions, and rewarding favorable interactions, resulting in a more reliable ranking of ligands. After rigorous evaluation, 125 compounds were selected for the final stage. The criteria included docking scores, presence of key hydrogen bonds or hydrophobic interactions, and favorable energy conformations.

Virtual Inspection and Physicochemical Profiling:

The top 125 compounds from the XP docking step underwent virtual inspection to assess their drug-likeness and potential absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. This involved the evaluation of physicochemical properties, such as molecular weight, logP, hydrogen bond donors and acceptors, and polar surface area. These factors were crucial for narrowing down the selection of 100 final compounds for biological testing. Compounds exhibiting optimal physicochemical profiles as well as those fitting the binding site were prioritized (Table 1).

Table 1: The top four compounds identified by virtual screening and physicochemical profiling and selected for biological testing

Compound ID	Compound Formula	Docking Score	Structure
ZINC000095356735	$C_{15}H_{25}N_5O_2$	-9.695	
ZINC000067908645	$C_{13}H_{19}N_7O$	-8.613	
ZINC000218827339	$C_{14}H_{14}FN_5O_2$	-8.583	
ZINC000091593654	$C_{15}H_{22}N_6O_3$	-8.113	

Final Compound Selection for Biological Testing:

The top four compounds identified by virtual screening and physicochemical profiling were selected for subsequent biological testing (Table 1). These compounds represent those

with the highest binding affinity for the DENV protein and the best overall ADMET profiles. The selected compounds were expected to undergo experimental validation to confirm their inhibitory activity against DENV1-4, focusing on their interactions with the 2FOM protein structure.

Cell Culture Reagents and Virus Strains:

Vero (African green monkey kidney) cells obtained from ATCC (CRL-1587) were maintained in Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific, 11-995-073) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) from Neuromics (FBS002), 1% L-glutamine (Fisher Scientific, 25-030-081), and 1% penicillin/streptomycin (Fisher Scientific, 15-140-122). *Aedes albopictus* clone C6/36, also from ATCC (CRL-1660), was grown in EMEM (ATCC, 30-2003) supplemented with 10% FBS. All cell lines were maintained at 37°C in 5% CO_2. Dengue virus type 2 (DENV2) was purchased from ATCC (VR-1584).

Cell Viability Assay:

About 1.25 x 10^5 Vero cells were seeded overnight in 1mL of DMEM per well of a 12 well plate. The Vero cells were treated with varying concentrations (100 µM, 50 µM, 20 µM, and 10 µM) of the compounds to be tested and allowed to incubate at 37°C in 5% CO_2. At 24, 48, and 72 h post-treatment, the supernatant was removed, and each well was washed once with sterile phosphate-buffered saline (PBS). The cells were trypsinized, transferred to a 1.5 mL microcentrifuge tube, and spun down to remove trypsin.

The cell pellet was resuspended in 500 µL of complete DMEM. For this experiment, 10 mM of the compound and DMEM was used as positive and negative control respectively. Each aliquot was mixed with stain-acridine orange/propidium iodide (AO/PI) at 1:9 ratio, and sample fluorescence was quantified using a Luna-FL cell counter (Logos Biosystems). Assays were performed in technical duplicates and biological triplicates (16).

Plaque Assay:

Vero cells were placed in a 12-well culture plate at a density of 1.5 × 10^5 cells per well and incubated for about 24 h to reach 90%-100% confluency. Culture supernatant from treatments containing DENV type 2 strain was serially diluted ten-fold in complete DMEM from 10^{-1} to 10^{-4} and 200 µL of diluted inoculum was used for Vero cells infection. After the cells were infected for approximately 2 h, the virus inoculum was removed, and 1 ml overlay of a 1:1 mixture of 0.6% agarose in deionized H_2O (diH_2O) and 2x unstained EMEM supplemented with 5% FBS, 1% Sodium Pyruvate, 2% penicillin/streptomycin, 1% nonessential amino acids and 1% L-glutamine was added to the inoculum in the wells.

The overlay was left at room temperature to solidify and was incubated for 7 days at 37°C in 5% CO_2. After 7 days, the cells were fixed with 10% formalin for 20 min at room temperature. The formalin and the agarose plugs were removed and subsequently, the cells were stained with the mixture of 1% crystal violet and 20% methanol in diH_2O solution for 30–60 s at room temperature (17).

RNA Isolation and Quantitative RT-PCR (RT-qPCR) Analysis of Viral Genome:

Total RNA was isolated from different samples using Direct-zol RNA Miniprep from Zymo research (R2052). The following reagents were obtained from BEI Resources, NIAID, NIH: Dengue Virus type 2 primers (NR-12079). Purification was performed following the manufacturer's protocol. For RT-qPCR, the Thermo Fisher RNA Ultra Sense One-Step Quantitative RT-PCR System (117 32927) was used for actin detection (Thermo Fisher; 401846), while the Power SYBR™ Green RNA-to-CT™ 1-Step Kit (Thermo Fisher- 4389986) was used to detect the DENV 2 region of the E gene (BEI resources; NR-12079). Primer pairs used for PCR were as follows; A) E gene: DENV2-F Polymerase Forward (5'–TCCATACACGCCAAACATGAA–3'), and DENV2-R Polymerase Reverse (5'–GGGATTTCCTCCCATGATTCC–3'); B) Actin: Forward (5'-CACCATTGGCAATGAGCGGTTC-3') and Reverse (5'-AGGTCTTTGCGGATGTCCACGT-3').

The PCR cycling conditions for the Power SYBR™ Green RNA-to-CT™ 1-Step Kit and the RNA Ultra Sense One-Step Quantitative RT-PCR System were performed according to the manufacturer's protocol. The RNA count of the samples was determined relative to the reference gene actin or based on the cycle threshold (Ct) value relative to the standard curve. All assays were performed in triplicate and biological triplicate.

Viral Replication Assay:

Approximately 5 × 10^5 Vero cells were seeded in a 12-well culture plate and incubated overnight at 37°C, 5% CO_2 to allow the cells to attach. The cells were then treated with different concentrations of compounds (500nM, 1 µM, 10 µM, and 20 µM) for 4 h. The compound was aspirated from the wells and washed three times using 1x PBS before subsequent infection with DENV2 at a multiplicity of infection (MOI) of 0.1 for 2h at 37°C, 5% CO_2. The inoculum was then removed, and the cells were washed twice with PBS and incubated in culture medium plus the compound at the respective concentrations for 48 h. Plaque assays and RT-qPCR were performed to determine viral load and viral genetic expression.

Assays were performed in technical duplicates and biological triplicates.

Statistical Analysis:

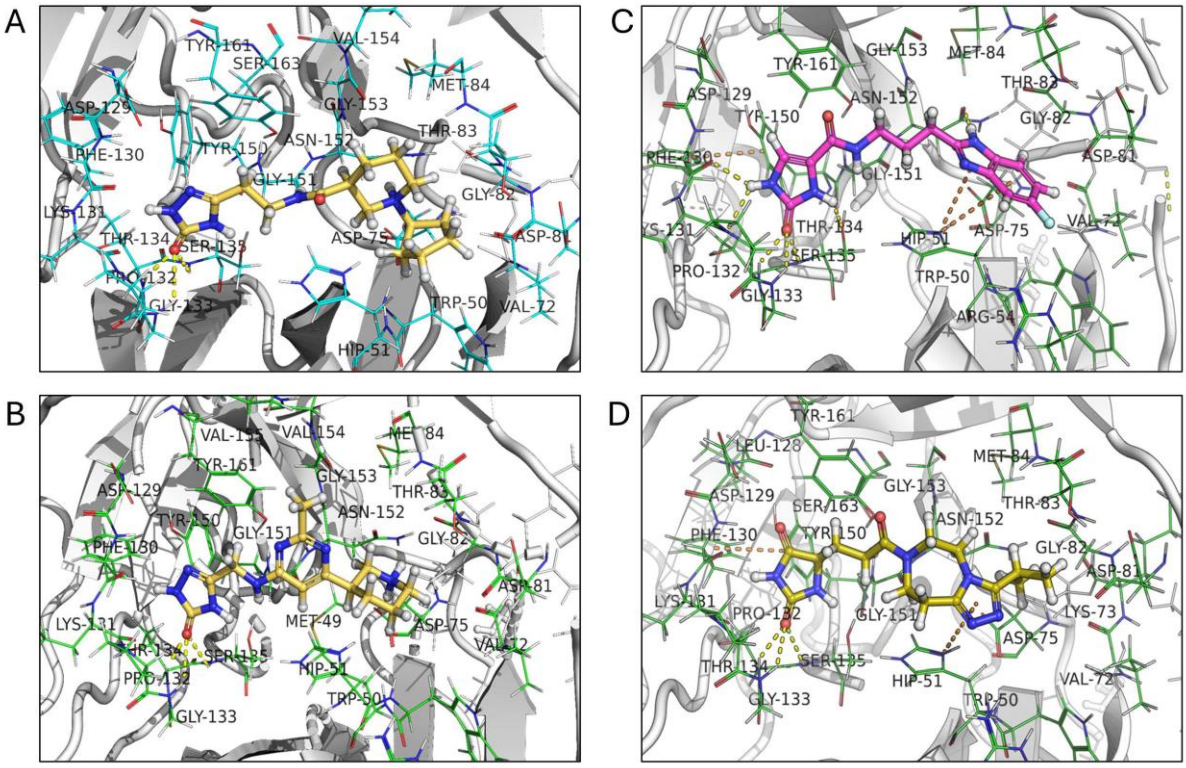
Statistical analysis was performed using GraphPad Prism, unless otherwise noted. For the parametric data, the significance was determined using the Student's *t*-test or one-way ANOVA and $p<0.05$.

Results:

Molecular docking of DENV protease:

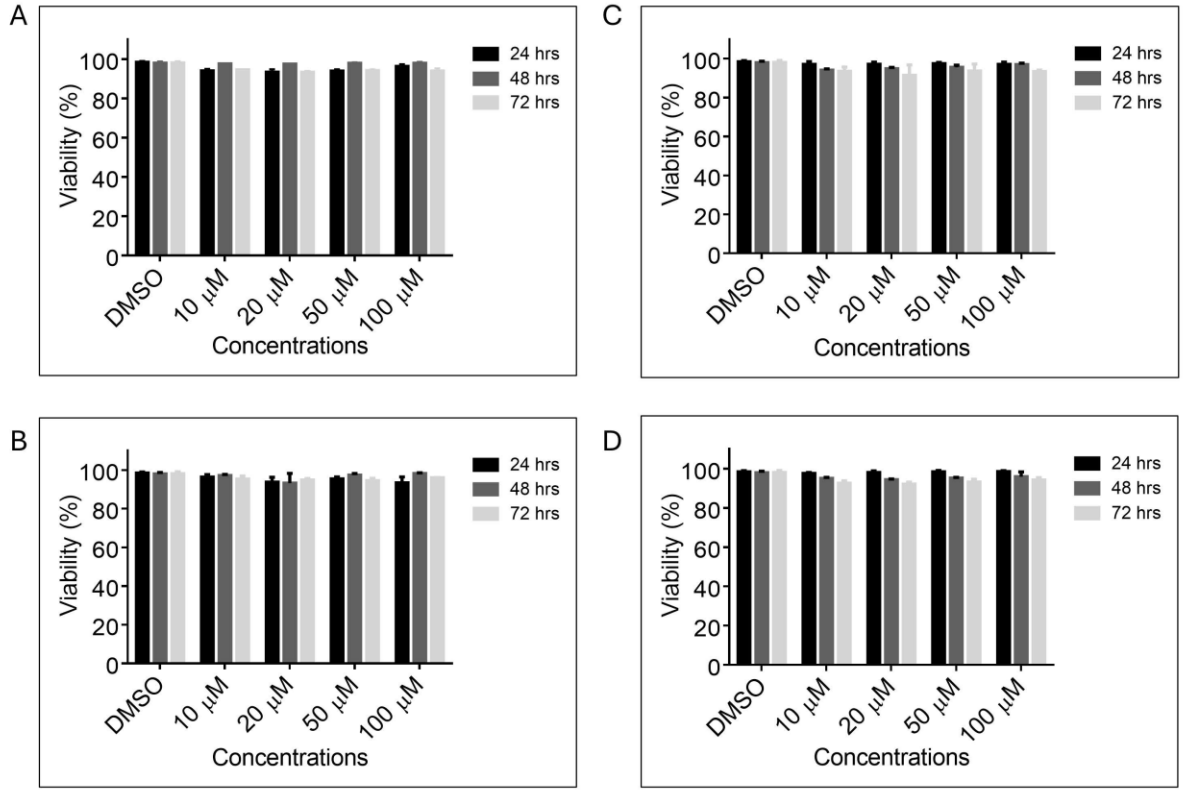
A dataset of over 1 million compounds was sourced from the 3D tranches dataset of the Zinc 21 database for virtual screening of the DENV protein (PDB ID: 2FOM). The compounds were first filtered based on their drug-likeness using Lipinski's Rule of Five to ensure their commercial availability. This process applies strict criteria such as molecular weight (250–500 Da), LogP (-1 to 4), and charge (-2 to +2) to focus on compounds with potential for drug development. Once the initial screening was complete, the selected compounds were downloaded as a PowerShell file, renamed as name_of_file.ps1, and converted into SDF (Structure Data File) format using PowerShell. This conversion enabled the compounds to be uploaded to Maestro, a docking program from Schrödinger LLC.

After importing the compounds into Maestro, further refinement was conducted using QikProp analysis to evaluate their ADME properties. To further prioritize the compounds, a STAR analysis was performed, ranking the compounds on a scale from 0 to 5 stars, with 0 stars as the cutoff for removal. Using these rigorous filtering steps and defined cutoffs for key ADME properties, the initial library of over a million compounds was narrowed down to four candidate compounds with strong drug-like characteristics, ready for further evaluation and testing in subsequent stages of the drug discovery process (Fig 1).



(A) ZINC95356735 (C, yellow; H, white; N, blue; O, red), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and green for all π interaction**. (B)** ZINC000067908645 (C, yellow; H, white; N, blue; O, red), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and green for brown for all π- interaction. (**C)** ZINC000218827339 (C, pink; H, white; N, blue; O, red; F, Cyan), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and green for brown for all π- interaction. (**D)** ZINC000091593654 (C, gold; H, white; N, blue; O, red), docked into DENV-2 (PDB: 2FOM), using the induced fit docking program (Schrödinger, LLC). Broken lines show interaction type; yellow for H-bonding and brown for all π- interaction. Docking pictures was generated with PyMOL molecular graphics system, version 2.0 Schrödinger, LLC.

Fig 1: The predicted binding interaction pose of zinc compounds

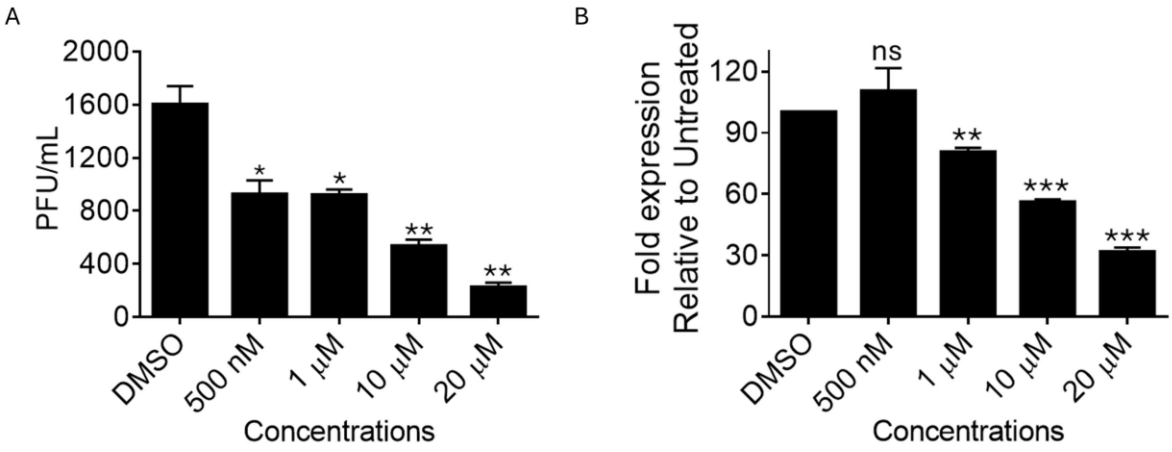


Naïve Vero cells were treated with increasing concentrations (10, 20, 50, and 100 μM) of compounds 1–4 (in Fig 1A - Fig 1D respectively), and cell viability was measured using acridine orange/propidium iodide (AO/PI) staining for up to 72 h post-treatment. DMSO was used as a control. The mean values ± SEM from three biological replicates are shown.

Fig 2: Cytotoxic effect of zinc compounds against Vero cells.

Compound show no cytotoxicity on Vero cell in the cell viability assay:

First, we assessed the cytotoxicity of the top four compounds against Vero cells. Cells were either treated with compounds or DMSO control for up to 72 h, after which viability was examined using acridine orange/ propidium iodide (AO/PI). Compound concentrations of up to 100 μM showed no significant cytotoxic effects in Vero cells after 72 h of incubation (Fig 2A–2D).



(A) Naïve Vero cells were treated with either compound 1 (500 nM, 1 μM, 10 μM and 20 μM) or DMSO control. After four hours, the cells were infected with DENV2 for 48 hours. Subsequently, supernatant samples were obtained and analyzed using plaque assay for viral load determination. (**B)** Naïve Vero cells were treated with either compound 1 (500 nM, 1 μM, 10 μM and 20 μM) or DMSO control. After four hours, the cells were infected with DENV2 for 48 hours. Subsequently, cell samples were obtained and analyzed using RT-qPCR to determine DENV2 genetic expression relative to DMSO control. For each treatment condition and time point assayed, mean values ± SEM from three biological replicates is shown. ns (no significance), *P ≤ 0.03; **P ≤ 0.01; ***P ≤ 0.005.

Fig 3: Pre-treatment of Naïve Vero Cells with Compound 1 Significantly Reduces Viral Replication in the Cells.

Pre-treatment of Vero cells with compound 1 reduces viral replication and viral release post-infection:

Compound 1 was analyzed for its potential role in reducing viral replication or release using Monkey Vero cells, which is an established cell infection model for DENV 2. We first treated the cells with different concentrations of the compounds (500 nM, 1 µM, 10 µM, and 20 µM) for 4 h, followed by infection with DENV 2. At 72 h post-infection, the supernatant samples were collected to determine viral release. The results shown in Fig 3A demonstrate that pretreatment with compounds at different concentrations resulted in a significant decrease in DENV release. An approximately two-fold decrease was observed at 500 nM, while an up to eight-fold decrease in viral release was observed at 20 µM (Fig 3A).

We also treated the cells with TRIzol to determine the intracellular viral load using RT-qPCR. Our results showed that while the 500 nM concentration did not show a statistically significant difference compared to the control, other higher concentrations showed a significant reduction in the intracellular viral load compared to the DMSO control (Fig 3B). These results collectively show that Compound 1 has antiviral activity against DENV 2.

Discussion:

Repurposing drugs is currently a major practice used to identify novel drug candidates (18,19,20,21). This strategy is based solely on knowledge of pharmacology and the clinical effects of the compound based on its original use (1,4,22). For this study, a dataset of over 1 million compounds was sourced from the 3D tranches dataset of the Zinc 21 database for virtual screening of the DENV protein (PDB ID: 2FOM). The compounds were first filtered based on their drug-likeness using Lipinski's Rule of Five to ensure their commercial availability. This process applies strict criteria such as molecular weight (250–500 Da), LogP (-1 to 4), and charge (-2 to +2) to focus on compounds with potential for drug development.

The compounds were streamlined down to 100 compounds based on criteria such as docking score. The top four compounds were selected based on their availability and docking scores for testing against DENV2. Our experiments using the Vero cell line as a model to test different compounds against DENV2 demonstrated that compound 1(2-(tert-butyl amino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide) robustly decreased the viral load along the concentration gradient (Fig 3), suggesting that the compound interferes with the viral replication process. We do not know yet if the interference is due to modulation of host response to Dengue infection or the direct inhibition of dengue protease activity. Understanding this will explain the antiviral activity observed.

Compound 1 is a c-Jun N-Terminal Kinase 1 (JNK1) inhibitor. The compound has been utilized to demonstrate efficacy against pulmonary fibrosis in animal models via oral administration (23,24). Therefore, it is currently being evaluated in phase II clinical trials for fibrosis (25). Although the mode of action of 2-(tert-butylamino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide is yet to be determined, as this is the first report of this compound to be used as a potential antiviral candidate against DENV2, we believe that the compound does not exert its major function to prevent viral entry. To this effect, further studies need to be carried out to determine the mechanistic framework of 2-(tert-butylamino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide. In addition to this, the remaining compounds should be tested for their antiviral properties against dengue virus infection.

The development of genetic alterations to control mosquito populations is an ongoing endeavor to control dengue infection. While this has very high potential, environmental concerns are a major factor affecting this approach (21,26). This with other concerns made anti-dengue drug development a top priority for the control of dengue infection. The challenges that occur during therapeutic drug development for the dengue virus include the existence of four DENV (1-4) serotypes, a lack of appropriate animal models, and the need for large trials because of different populations and clinical outcomes (13). Therefore, it is important to identify potential candidates to mitigate these challenges.

Conclusion:

In this pilot study, we tested the antiviral activity of the top compound (2-(tert-butylamino)-4-[[(1R,3S)-3-hydroxycyclohexyl]amino]pyrimidine-5-carboxamide) from an in-silico study against type 2 dengue virus NS2B/NS3 protease (PDB ID: 2FOM) and observed significant antiviral activity against DENV2. This shows a significant step towards the discovery of an antiviral drug against the dengue virus. It is important to note that this is a preliminary pilot study, and more work needs to be done to know the mechanism of action of this compound. Also, dengue virus serotypes share conserved regions, and future work should check if this compound is able to produce antiviral activity against other serotypes.

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Contributions of authors:

All authors contributed significantly to the manuscript. AAO and TEE designed the experiment. AAO, SJR, NU, KG, PEG and OQ were involved in the analysis. AAO and TEE drafted and reviewed the write-up. All authors critically reviewed the final manuscript.

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Conflict of interest:

Authors declare no conflict of interest.

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